Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1α, MIP-1β, and RANTES

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Abstract: We have cloned a human cDNA for a novel CC chemokine receptor (CC CKR) designated CC CKR5 that has 48–75% amino acid identity to other CC CKRs. CC CKR5 mRNA was detected constitutively in primary adherent monocytes but not in primary neutrophils or eosinophils. Macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES were all potent agonists for CC CKR5 (EC_{50} = 3–30 nM) when calcium flux was measured in transfected HEK 293 cells, yet the apparent binding affinities of the corresponding iodinated chemokines to intact cells expressing the receptor were low (IC_{50} ~100 nM). The calcium flux responses were completely blocked by treatment of transfected cells with pertussis toxin. These data suggest that CC CKR5 is a G_i-coupled receptor that may mediate monocyte responses to MIP-1α, MIP-1β, and RANTES. *J. Leukoc. Biol.* 60: 147–152; 1996

Key Words: chemotaxis · inflammation · G protein

INTRODUCTION

Local accumulation of leukocytes is a characteristic feature of the inflammatory response to infectious and other noxious agents, however, the specific types of leukocytes that accumulate depend on the inciting agent and can vary widely. The factors determining this specificity are not fully known but probably include members of the chemokine superfamily. The CC branch of the chemokine superfamily includes macrophage inflammatory protein-1α (MIP-1α), MIP-1β, RANTES, I-309, monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, and eotaxin. They have highly conserved amino acid (aa) sequences, including four conserved cysteine residues, the first two of which are adjacent. All of these molecules except for eotaxin induce monocyte chemotaxis but they vary both in monocyte chemotactic potency and in their ability to attract and activate neutrophils, lymphocytes, basophils, and eosinophils [1–9]. Eotaxin is highly selective for eosinophils [10–12].

Specific monocyte binding sites for MIP-1α, MIP-1β, RANTES, MCP-1, and MCP-3 have been identified, and molecular cloning has identified a family of distinct but related genes expressed in monocytes that encode receptors with overlapping selectivity for MIP-1α, RANTES, MCP-1, and MCP-3 [2, 13–22]; their deduced amino acid sequences have >45% aa identity to each other. Three of us recently reported the sequence of a human eosinophil receptor designated CC CKR3 that is expressed at much lower levels in monocytes. CC CKR3 was originally reported to be selective for MIP-1α, MIP-1β, and RANTES based on calcium flux responses in transfected HEK 293 cells. We later retracted this claim, having discovered that the cells thought to have been transfected with the CC CKR3 plasmid were inadvertently transfected with a related cDNA encoding a novel CC chemokine receptor that we have designated CC CKR5 [ref. 23 and correction]. This mistake occurred because the CC CKR5 plasmid DNA was mislabeled CC CKR3. We later reported that CC CKR3 is selective for eotaxin and not other CC chemokines tested [12].

Samson et al. have recently reported the cloning of a human gene that encodes a human CC chemokine receptor selective for MIP-1α, MIP-1β, and RANTES, also named CC CKR5, that differs from our CC CKR5, sequence only at amino acid 90 [24]. Here we characterize in detail our CC CKR5 variant. This work extends the work of Samson et al. by identifying the first cDNA for CC CKR5, by describing a novel allelic variant of CC CKR5, by detailing its ligand binding properties and RNA distribution in primary leukocyte subtypes, and by demonstrating that it couples to a pertussis toxin-sensitive signal transduction pathway.

MATERIALS AND METHODS

Cloning of the CC CKR5 cDNA

The methods used to clone novel chemokine receptor-like cDNAs from a λgt11 cDNA library made from peripheral blood mononuclear cells of

Abbreviations: MIP-1α, macrophage inflammatory protein-1α; MCP, monocyte chemoattractant protein; HEK, human embryonic kidney; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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a patient with eosinophilic leukemia have been described previously [20]. One of the cDNAs obtained, designated clone 63-2, had a novel sequence highly related to CC CKR2B, but it extended only from bp 105 to 813 of the CC CKR2B ORF. The 63-2 cDNA was then used as a hybridization probe to screen under low stringency conditions (final wash: 5× SSPE, 55°C for 30 min) a λpCEV9 cDNA library prepared from endotoxin-stimulated human peripheral blood monocytes as described previously [20, 23]. One of the isolated clones, designated clone 8.5, matched and extended the sequence of clone 63-2. A 1.4-kb fragment of the clone 8.5 cDNA was excised from the vector DNA by *Bam* HI and *Bst* XI double digestion, blunt-ended with *Pfu* DNA polymerase, subcloned into the *Eco* RV site of pBluescript II KS (Stratagene, La Jolla, CA), and sequenced completely on both strands. The cDNA insert was then subcloned between the *Bam* HI and *Hind* III sites of the mammalian expression vector pREP9 (Invitrogen, San Diego, CA). Human embryonic kidney (HEK) 293 cells (10^7) grown to log phase in Dulbecco's modified Eagle's medium and 10% fetal bovine serum were electroporated with 20 μg of plasmid DNA, and G418-resistant colonies were picked and expanded as described [23]. The methods used to create HEK 293 cell lines stably expressing CC CKR1 and CC CKR2B have been described [21].

RNA analysis

Peripheral blood leukocyte RNA was prepared and analyzed by hybridization to the indicated cDNA probes using methods described previously [20]. An antisense 30-mer oligonucleotide 5′-GTCATAGATTGGACTTGACACTTGATAATC (nucleotides +4 to +33 of the clone 8.5 cDNA, where +1 is the adenine in codon 1) that does not bind to CC CKR1, CC CKR2, or CC CKR3 DNA or RNA was used for Northern blot analysis using conditions described previously [21].

Ligand binding analysis

Transfected HEK 293 cells (10^6) were incubated in duplicate with 0.2 nM ^{125}I-labeled RANTES, MCP-1, MIP-1α, MIP-1β, or MCP-3 (specific activity ~2200 Ci/mmol, Du Pont/New England Nuclear, Boston, MA) and varying concentrations of unlabeled recombinant human chemokines (Peprotech, Rocky Hill, NJ) in 200 μL of binding medium [RPMI-1640 with 1 mg/mL bovine serum albumin (BSA) and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4]. After incubation for 1 h at 37°C, unbound chemokines were separated from cells by pelleting through a 10% sucrose/phosphate-buffered saline (PBS) cushion.

Receptor activation assay

$[Ca^{2+}]_i$ changes were measured using 2 million transfected HEK 293 cells loaded with Fura-2 upon stimulation with chemokines as described previously [21]. Where indicated, cells loaded with Fura-2 were incubated in holotoxin of *B. pertussis* (List, Campbell, CA) 250 ng/mL for 2 h at 37°C, then washed twice in PBS and resuspended in Hanks' balanced saline solution. Cell viability was ~80% by trypan blue exclusion after pertussis toxin treatment. ATP was from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

Cloning and sequence analysis of CC CKR5

The clone 8.5 cDNA for CC CKR5 is 1370 bp in length. The 5′- and 3′-untranslated regions and ORF are 26, 288, and 1056 bp, respectively. The 3′-untranslated region is not polyadenylated and lacks a polyadenylation consensus sequence. The ATG codon proposed to initiate translation is flanked by sequence that conforms favorably with the consensus rules established by Kozak [25]. The ORF contains 352 codons. The sequence is 57, 70, 75, 51, and 48% identical to CC CKR1, CC CKR2A, CC CKR2B, CC CKR3, and CC CKR4, respectively. Only a few very small gaps are required for optimal alignment (Fig. 1).

Like other seven-transmembrane-domain receptors, the C-terminal tail has a high content of serine and threonine residues that may be sites for receptor phosphorylation as they are in rhodopsin and the β2-adrenergic receptor [26]. It also contains cysteine residues that by analogy with other seven-transmembrane domain receptors could be a site for palmitoylation, tethering this domain to the plasma membrane [27]. The net charge of the *N*-terminal extracellular segment of CC CKR5 is -1. The corresponding domain of CC CKR2B has a net charge of zero, whereas for other known chemokine receptors this domain is highly acidic [28]. Like all other known chemokine receptors, CC CKR5 has conserved cysteine residues in the *N*-terminal segment and the third predicted extracellular loop that could form a disulfide bond. CC CKR5 has a consensus sequence for *N*-linked glycosylation, in the predicted third extracellular loop. Compared with the sequence of Samson et al., our sequence contains a leucine in place of alanine at position 90 [24]. The two sequences probably derive from distinct alleles of the same gene.

Distribution of CC CKR5 RNA

Samson et al. screened several cultured leukocyte cell lines and found RNA for CC CKR5 only in KG-1A promyeloblastic cells [24]. Cross-hybridization to CC CKR2 mRNA, which is similar in size to the transcript found, was not excluded in this study, and analysis of primary peripheral blood mononuclear cell RNA was reported as negative. In our experiments, a full-length CC CKR5 ORF probe clearly recognized a 3.5-kb RNA band by Northern blot hybridization in total RNA made from primary adherent monocytes, but not from primary neutrophil or eosinophil samples (Fig. 2, left). We next synthesized a 30-mer antisense CC CKR5 oligonucleotide that does not cross-hybridize with CC CKR2 and used it to probe the same blot. The same band was identified by this probe, confirming monocyte expression of CC CKR5 (Fig. 2, right).

Agonists for CC CKR5

As described in the Introduction, Figures 3 and 4 of our previously published study showing calcium flux responses in CC CKR5-transfected HEK 293 cells stimulated with MIP-1α, MIP-1β, and RANTES but not MCP-1, MCP-2, or MCP-3 were incorrectly attributed to CC CKR3 [23]. Samson et al. have also reported that MIP-1α, MIP-1β, and RANTES, but not MCP-1 or MCP-3 were agonists for their allelic variant of CC CKR5 when pH changes were measured as the functional response in stably transfected CHO-K1 cells. We have extended these studies by showing that treatment of CC CKR5 transfectants with pertussis toxin completely abolished the calcium flux response to MIP-1α, MIP-1β, and RANTES (Fig. 3). In contrast, the calcium

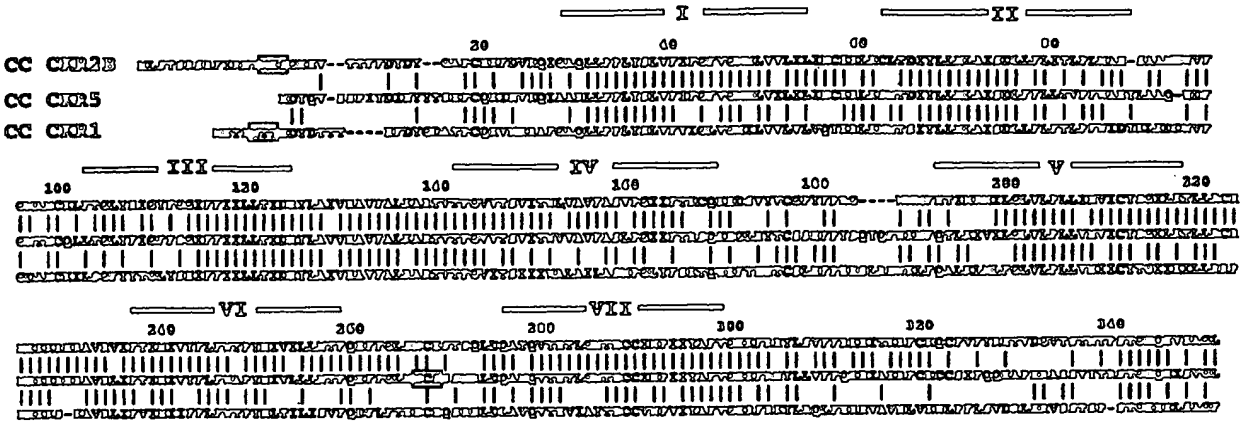

Fig. 1. Alignment of the amino acid sequence deduced from cDNAs for CC CKR1, CC CKR2B, and CC CKR5. Arabic numerals correspond to the CC CKR5 sequence and are left-justified. Putative membrane-spanning segments I-VII are noted. Vertical lines, identities between adjacent residues; open boxes, predicted sites for *N*-linked glycosylation; dashes, gaps inserted to optimize the alignment. The sequence has been deposited in GenBank, accession number U57840.

flux response to ATP, which probably acts by binding to endogenous purinergic receptors, was largely unaffected.

The potency order reported by Samson et al., MIP-1α > RANTES = MIP-1β, differs slightly from that reported for our CC CKR5 variant using a calcium flux assay as the functional response: MIP-1α > RANTES > MIP-1β. The concentration for half-maximal responses to MIP-1α was ~3 and 10 nM, respectively, in both studies. In our study MIP-1β was approximately threefold less potent than RANTES, whereas, in the study of Samson et al., the dose-response curves for RANTES and MIP-1β were very similar [23, 24]. It should be appreciated that the differences among the three agonists and between the two studies in both potency and efficacy are quite small and could result from any one of a number of differences in the materials and methods used, including the CC CKR5 variant tested, the levels of expression achieved, differences between CHO-K1 and HEK 293 cells, the source of chemokines, and variability from experiment to experiment using the same system.

Binding of CC chemokines to CC CKR5

In the description of their CC CKR5 variant, Samson et al. were unable to show specific binding of ^{125}I-MIP-1α to stably transfected CHO-K1 cells at 4°C [24]. When we tested our HEK 293 transfectants expressing high levels of CC CKR5 activity, we observed low levels of specific ^{125}I-MIP-1α binding at 4°C, whereas much higher levels of binding were found for cells expressing CC CKR1 (not shown). However, specific binding of ^{125}I-labeled MIP-1α, MIP-1β, and RANTES was more readily detected on our CC CKR5 transfectants when binding assays were carried out at 37°C (Fig. 4). The same radioligands did not bind specifically to untransfected HEK 293 cells or CC CKR2B-transfected HEK 293 cells at 37°C, whereas the latter bound both ^{125}I-MCP-1 and ^{125}I-MCP-3, as expected (data not shown).

^{125}I-MCP-1 did not bind specifically to either CC CKR1 or CC CKR5 transfectants; ^{125}I-MCP-3 bound specifically to CC CKR1, confirming previous reports [21, 22], but not

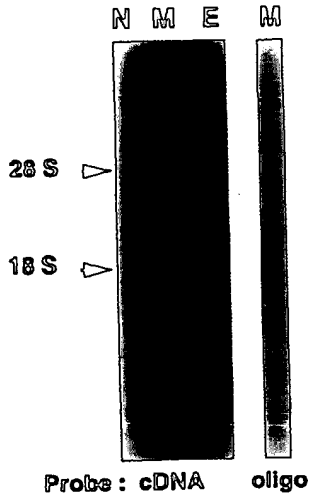

Fig. 2. CC CKR5 mRNA distribution in human leukocyte subsets. A Nytran blot containing 10 μg total RNA from peripheral blood-derived human neutrophils (N), adherent monocytes (M), and eosinophils (E) was hybridized with the clone 8.5 cDNA encoding CC CKR5. The blot was washed at 65°C in 0.2× SSPE for 1 h, then exposed to XAR-2 film with an intensifying screen at -80°C for 2 days. Lanes N and E were positively controlled with IL8RB and CC CKR3 probes, respectively (not shown). A separate lane containing 10 μg adherent monocyte RNA was hybridized with a 30-mer antisense oligonucleotide probe specific for CC CKR5 and was washed in 5× SSPE at 50°C for 15 min. The result shown is after 2 weeks of exposure to XAR-2 film. The position of ribosomal bands is indicated at the left.

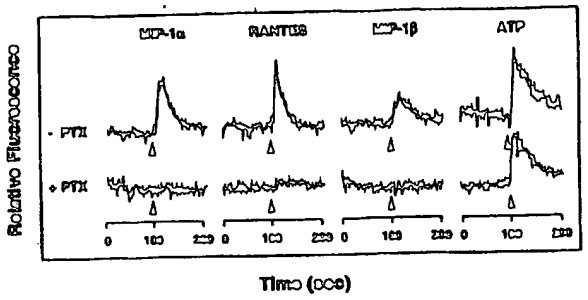

Fig. 3. G protein coupling to CC CKR5. $[Ca^{2+}]_i$ was measured as the relative fluorescence emitted by HEK 293 cells stably transfected with CC CKR5, loaded with Fura-2, and treated with pertussis toxin or vehicle. Each tracing represents the results of a separate cuvette containing 2 million cells stimulated at the time indicated by the arrow with the CC CKR5 agonist indicated above the corresponding column of tracings. The top and bottom rows of tracings correspond to cells treated with vehicle (-PTX) and pertussis toxin (+PTX), respectively. Chemokines were tested at 100 nM; ATP was tested at 5 μM.

to the CC CKR5 transfectants (data not shown). These results are consistent with the agonist specificity of the two receptors. The binding of ^{125}I-MIP-1α and ^{125}I-MIP-1β to CC CKR1- and CC CKR5-transfected cells at 37°C could be easily distinguished in two ways. First, binding of both ^{125}I-MIP-1α and ^{125}I-MIP-1β to CC CKR1 could be competed effectively by unlabeled MCP-3 (>75% competition at 500-fold molar excess), whereas MCP-3 did not compete for binding of either chemokine to CC CKR5 when tested at 500-fold molar excess. This pattern is consistent with the agonists for CC CKR1 and CC CKR5. Second, MIP-1α competed ~20-fold more effectively for ^{125}I-MIP-1α binding to CC CKR1 than to CC CKR5 (half-maximal inhibitory concentrations [IC_{50}] ~5 and 100 nM, respectively), and unlabeled MIP-1β competed approximately twofold more effectively for ^{125}I-MIP-1β binding to CC CKR5 than to CC CKR1 (IC_{50}s ~100 and 200 nM, respectively; Fig. 4, A and B). The results suggest that MIP-1α binds with substantially lower affinity to CC CKR5 than to CC CKR1, whereas MIP-1β binds with similar low affinity to both receptors. In this regard, it is interesting that MIP-1α has similar potency as an agonist for both receptors, whereas MIP-1β is a much less potent and effective agonist for CC CKR1 than for CC CKR5.

^{125}I-RANTES could also bind specifically to both CC CKR1 and CC CKR5 transfectants (Fig. 4C). We had difficulty demonstrating the specificity of ^{125}I-RANTES binding using excess unlabeled RANTES, as have others [16, 22], whereas MIP-1α clearly competed for ^{125}I-RANTES binding. The ^{125}I-RANTES binding sites at CC CKR1 and CC CKR5 could be distinguished by heterologous competition with excess unlabeled MIP-1α (IC_{50}s ~20 and 100 nM for CC CKR1 and CC CKR5, respectively; Fig. 4C). The high IC_{50} values for binding compared with the agonist EC_{50} values for CC CKR5 suggest that its ability to transduce signals is very efficient. Additional studies will be needed to determine whether the apparent low-affinity binding of chemokine agonists to CC CKR5 is due to structural differences from iodination or differences in G proteins between monocytes and HEK 293 cells. However, it is important to note that both the calcium flux assay and the radioligand binding assays applied to CC CKR5-transfected cells gave the same chemokine specificity, positive for MIP-1α, MIP-1β, and RANTES, negative for other chemokines tested.

Although CC CKR2B and CC CKR5 are the most structurally similar CC chemokine receptors, having 75% aa identity, they are least related in function, having no common agonists. In contrast, MCP-1 is an agonist for both CC CKR2B and CC CKR4, and MCP-3 is an agonist for CC CKR2B and CC CKR1, even though these pairs of recep-

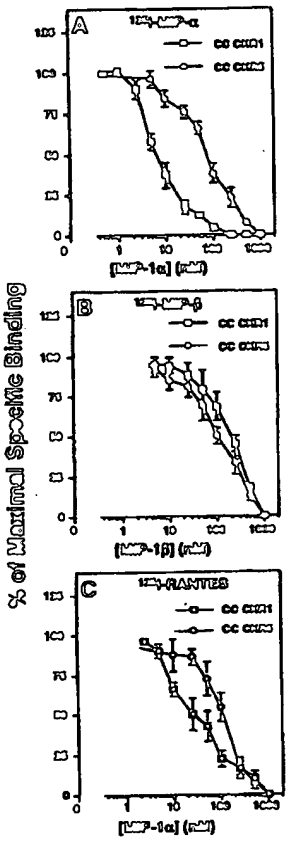

Fig. 4. Radioligand binding properties of CC CKR1 and CC CKR5. HEK 293 cells stably transfected with CC CKRs were incubated at 37°C with 0.2 nM of the radioligands (200,000 cpm) indicated at the top of each box. (A–C) % maximal specific binding plotted as a function of increasing amounts of unlabeled MIP-1α (A and C) or MIP-1β (B). Maximal specific binding is defined as the difference between total cell-associated counts in the absence of unlabeled chemokine and in the presence of 1000 nM of the unlabeled chemokine indicated on the abscissa of each panel. For CC CKR1 and CC CKR5, respectively, nonspecific binding was as follows: ^{125}I-MIP-1α, 3 ± 2 and 31 ± 6%; ^{125}I-MIP-1β, 37 ± 5 and 22 ± 6%; and ^{125}I-RANTES, 65 ± 2 and 58 ± 2%. Data are pooled from 3–5 separate experiments for (A) and (B), and from 2 separate experiments for (C).

tors have only 47 and 57% aa identity, respectively [16–19, 21, 22, 30]. Surprising structure-function relationships have previously been identified for the two human CXC chemokine receptors, interleukin-8 (IL-8) receptors A and B, which are 78% identical in amino acid sequence [31, 32]. IL8RB is selective for IL-8 and at least two other related CXC chemokines, whereas IL8RA is monoselective for IL-8. The *N*-terminal segment, and the second and third extracellular loops of IL8RB, are all dominant selectivity determinants when tested separately in IL8RA-IL8RB chimeric receptors [33]. Analysis of chimeric receptors may also be useful in identifying selectivity determinants for CC CKR2B and CC CKR5.

CC CKR5 is the first receptor identified for which MIP-1β is a potent agonist. In addition to CC CKR5, MIP-1α and RANTES are also agonists for CC CKR1 and CC CKR4, which are also expressed in monocytes [16, 17, 19, 20]. Additional studies using blocking reagents specific for individual receptor subtypes will be needed to define the contribution of each of these receptors to the monocyte-directed actions of MIP-1α and RANTES. Monocytes are long-lived cells capable of further differentiation as they move from the blood to establish residence in the tissues as macrophages. The functional properties of tissue macrophages can differ in different organs, and in the same organ depending on the presence of priming agents. In this regard, it will be important to determine whether monocyte CC CKRs are differentially expressed on subsets of monocytes or in tissue macrophages from different organs, and whether other CC CKRs exist that are expressed in these cells. Additional work will also be needed to address the role of each of the CC CKRs in other biological phenomena such as lymphocyte chemotaxis and hematopoiesis [34, 35] that are regulated by their ligands. Finally, it is important to note that the ligands for CC CKR5 are the same three molecules that have been shown to act as suppressors of replication for certain strains of HIV-1, making CC CKR5 the best-known candidate to mediate this activity [36].

Note added in proof: CC CKR5 is a fusion cofactor for macrophage-tropic strains of HIV-1 (Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996) *Science*. In press.

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